

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
NEW PATENT APPLICATION**

TITLE: AMINO-TERMINALLY TRUNCATED RANTES AS  
CHEMOKINE ANTAGONISTS

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AMINO-TERMINALLY TRUNCATED RANTES AS CHEMOKINE  
ANTAGONISTS

FIELD OF THE INVENTION

The present invention relates to amino-terminally truncated RANTES, lacking  
5 NH<sub>2</sub>-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3 or 1-4 of  
the naturally-occurring RANTES and having chemokine antagonistic activity, as well as  
cDNA sequences encoding them, their use in therapy and/or in diagnosis of the diseases,  
in which an antagonistic activity of the chemokine effects is required, and  
pharmaceutical compositions comprising them.

BACKGROUND OF THE INVENTION

Chemokines constitute a family of small pro-inflammatory cytokines with  
leukocyte chemotactic and activating properties. Depending on the position of the first  
cysteines, the chemokine family can be divided in C-C, C-X-C and C-X<sub>3</sub>-C chemokines  
15 (Baggiolini M. et al., 1994; Baggiolini M. et al., 1997 and Taub D. et al., 1996).

Many C-X-C chemokines such as interleukin-8 (IL-8) are chemotactic for  
neutrophils, while C-C chemokines, such as monocyte chemotactic protein-3 (MCP-3),  
are active on a variety of leukocytes including monocytes, lymphocytes, eosinophils,  
basophils, NK cells and dendritic cells.

20 The NH<sub>2</sub> terminal domain of chemokines is involved in receptor-binding and  
NH<sub>2</sub>-terminal processing can activate chemokines, reduce their chemokine activity or  
render chemokines completely inactive.

The C-X-C chemokine platelet basic protein becomes a neutrophil chemotactic  
peptide (NAP-2) only after removal of the 24 NH<sub>2</sub>-terminal residues (Walz A. et al.,  
25 1989 and Van Damme J. et al., 1990).

Deletion of up to 8 NH<sub>2</sub>-terminal residues from IL-8 results in an enhanced  
chemotactic activity, but further cleavage of the Glu-Leu-Arg motif, which is located in  
front of the first Cys in all neutrophil chemotactic C-X-C chemokines, causes complete  
inactivation (Clark-Lewis I. et al., 1991).

Similar NH<sub>2</sub>-terminal proteolysis (up to 8 amino acids) of another C-X-C chemokine, granulocyte chemotactic protein-2 (GCP-2), has no effect on the neutrophil chemotactic activity (Proost P. et al, 1993a).

5 RANTES (is an acronym for "Regulated upon Activation, Normally T Expressed, and presumably Secreted") is a C-C chemokine, whose cDNA clone has been isolated from a cDNA library enriched for T cell-specific sequences (Schall T. J. et al., 1988).

The synthetical C-C chemokines MCP-1, MCP-3 and RANTES missing the 8 to 9 NH<sub>2</sub>-terminal amino acids are inactive on monocytes and are useful as receptor antagonists (Gong J. et al., 1996; and Gong J. et al., 1995).

10 Extension of RANTES with one methionine results in complete inactivation of the molecule and Met-RANTES behaves as an antagonist for the authentic RANTES (Proudfoot A.E. et al., 1996).

#### DESCRIPTION OF THE INVENTION

15 The main object of the present invention is amino-terminally truncated RANTES, lacking NH<sub>2</sub>-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3 or 1-4 of the naturally-occurring RANTES and having chemokine antagonistic activity.

A particular object of the present invention is RANTES(3-68), which is RANTES lacking the first 2 amino acids, as shown in Figure 1 and in SEQ ID NO: 2.

20 The amino-terminally truncated RANTES of the invention can be in a glycosylated or non-glycosylated form.

The term "chemokine antagonist" means 'which acts as antagonist to the mature full-length naturally-occurring chemokines'.

25 Another object of the invention are the DNA molecules comprising the DNA sequences coding for the amino-terminally truncated RANTES of the invention, including nucleotide sequences substantially the same. The cDNA sequence of intact RANTES is disclosed in Schall T. J. et al. (1988) and the cDNA of the truncated RANTES can be easily deduced.

"Nucleotide sequences substantially the same" includes all other nucleic acid

sequences which, by the degeneracy of the genetic code, also code for the given

The invention also includes expression vectors which comprise the above DNAs, host-cells transformed with such vectors and a process of preparation of such aminoterminally truncated RANTES of the invention, through the culture in appropriate culture media of said transformed cells.

5       The DNA sequence coding for the proteins of the invention can be inserted and ligated into a suitable plasmid. Once formed, the expression vector is introduced into a suitable host cell, which then expresses the vector(s) to yield the desired protein.

10       Expression of any of the recombinant proteins of the invention as mentioned herein can be effected in eukaryotic cells (e.g. yeasts, insect or mammalian cells) or prokaryotic cells, using the appropriate expression vectors. Any method known in the art can be employed.

15       For example the DNA molecules coding for the proteins obtained by any of the above methods are inserted into appropriately constructed expression vectors by techniques well known in the art (see Sambrook et al, 1989). Double stranded cDNA is linked to plasmid vectors by homopolymeric tailing or by restriction linking involving the use of synthetic DNA linkers or blunt-ended ligation techniques: DNA ligases are used to ligate the DNA molecules and undesirable joining is avoided by treatment with alkaline phosphatase.

20       In order to be capable of expressing the desired protein, an expression vector should also comprise specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding the desired protein in such a way as to permit gene expression and production of the protein. First in order for the gene to be transcribed, it must be preceded by a promoter recognizable by RNA polymerase, to which the polymerase binds and thus initiates the transcription process.  
25       There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters).

For eukaryotic hosts, different transcriptional and translational regulatory sequences may be employed, depending on the nature of the host. They may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus or the like, or from cellular sources associated with a particular gene which has a high level

promoter, the yeast gal4 gene promoter, etc. Transcriptional initiation regulatory signals may be selected which allow for repression and activation, so that expression of the genes can be modulated.

5 The DNA molecule comprising the nucleotide sequence coding for the protein of the invention is inserted into vector(s), having the operably linked transcriptional and translational regulatory signals, which is capable of integrating the desired gene sequences into the host cell.

10 The cells which have been stably transformed by the introduced DNA can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may also provide for phototrophy to a auxotrophic host, biocide resistance, e.g. antibiotics, or heavy metals such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of proteins of the  
15 invention.

Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells, that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to  
20 "shuttle" the vector between host cells of different species.

Once the vector(s) or DNA sequence containing the construct(s) has been prepared for expression the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection,  
25 etc.

Host cells may be either prokaryotic or eukaryotic. Preferred are eukaryotic hosts, e.g. mammalian cells, such as human, monkey, mouse, and Chinese hamster ovary (CHO) cells, because they provide post-translational modifications to protein molecules, including correct folding or glycosylation at correct sites. Also yeast cells can carry out  
glycosylation. A number of

copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides).

After the introduction of the vector(s), the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the desired proteins.

The amino-terminally truncated RANTES of the invention may be prepared by any other well known procedure in the art, in particular, by the well established chemical synthesis procedures, utilizing automated solid-phase peptide synthesizers followed by chromatographic purification.

The chemokines of the invention may, for example, be synthesized by Fmoc (9-fluorenylmethoxycarbonyl), tBoc (t-butoxycarbonyl) or any other comparable chemical synthesis with or without appropriate side-chain protection groups on the different amino acids. The amino acids with or without appropriate side-chain protection groups are preactivated - e.g. with HBTU/HOBt [2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluromium hexafluorophosphate/1-hydroxybenzotriazole] - and coupled to the growing peptide chain. Before the addition of the following residue, the protection group (e.g. Fmoc) is removed from the  $\alpha$ -amino group. After synthesis, all protection groups are removed, the intact full length peptides are purified and chemically or enzymatically folded (including the formation of disulphide bridges between cysteines) into the corresponding chemokines of the invention.

Purification of the natural, synthetic or recombinant proteins is carried out by any one of the methods known for this purpose, i.e. any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like (see for example Proost P. et al., 1996). A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using monoclonal antibodies, or affinity for heparin, which bind the target protein and which are produced and immobilized on a gel matrix contained within a column. Impure preparations containing the proteins are passed through the column. The protein will be bound to the

antibody or heparin, while impurities will pass through. After washing the

The amino-terminally truncated RANTES of the invention are useful in the therapy and/or diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required. Examples of such diseases include: inflammatory diseases, angiogenesis- and hematopoiesis-related diseases, tumors, infectious diseases, including  
5 HIV, auto-immune diseases, atherosclerosis, pulmonary diseases and skin disorders. The preferred use is in the field of HIV-infection.

Therefore, in a further aspect, the present invention provides the use of the protein of the invention in the manufacture of a medicament for the treatment of the above-mentioned diseases.

10 The medicament is preferably presented in the form of a pharmaceutical composition comprising the proteins of the invention together with one or more pharmaceutically acceptable carriers and/or excipients. Such pharmaceutical compositions form yet a further aspect of the present invention.

A further embodiment of the invention is the method of treatment of the above-  
15 mentioned diseases comprising administering a pharmacologically active amount of the amino-terminally truncated RANTES of the invention to subjects at risk of developing such diseases or to subjects already showing such pathologies.

It has also been found that CD26/DPP IV is able to generate NH<sub>2</sub>-terminally truncated RANTES in vitro. RANTES is the first cytokine reported whose biological  
20 activity can be modified by CD26/DPP IV.

Therefore, another object of the present invention is the use of CD26/DPP IV in the therapy and/or diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required, with particular focus on inflammatory, immune and infectious diseases.

25 Since this represents the first example of an identified mechanism for endogeneously regulated chemokine modification into an antagonist, similar physiological processing, but mediated by other factors (proteases). The use of such factors (proteases) in the therapy and/or diagnosis of the above diseases is also included in this invention.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention. The Examples will refer to the Figures specified here below.

5 DESCRIPTION OF THE FIGURES

Figure 1: it shows the amino acid sequences of RANTES. Signal sequences are reported in *italics*, whereas C -residues are in **bold**. Arrows indicate the first amino acids of the amino-terminally truncated RANTES of the invention, also called RANTES(3-68).

10 Figure 2: The chemotactic potencies of intact and NH<sub>2</sub>-terminally truncated forms of natural or recombinant RANTES for monocytic THP-1 cells were compared in the Boyden microchamber assay: Natural RANTES(1-68) ( $\Delta$ ), natural, truncated RANTES(3-68) ( $\square$ ), intact recombinant RANTES(1-68) ( $\blacktriangle$ ) and CD26/DPP IV cleaved recombinant RANTES(3-68) ( $\blacksquare$ ). Results represent the mean chemotactic index  $\pm$  SEM of four or more independent experiments.

15 Figure 3: Effect of natural RANTES(3-68) ( $\square$ ), natural RANTES(1-68) ( $\Delta$ ), recombinant RANTES(1-68) ( $\blacktriangle$ ) and recombinant CD26/DPP IV treated RANTES(3-68) ( $\blacksquare$ ) on the  $[Ca^{2+}]_i$  in THP-1 cells. Results represent the mean increase in  $[Ca^{2+}]_i \pm$  SEM of three or more independent experiments.

20 Figure 4: It shows the desensitization of the  $Ca^{2+}$ -mobilizing activity of intact recRANTES(1-68) by RANTES(3-68). THP-1 cells were first stimulated with buffer or different concentrations of recombinant RANTES(1-68) or RANTES(3-68). Results represent the mean  $\pm$  SEM (three or more independent experiments) increase in  $[Ca^{2+}]_i$  in response to 30 ng/ml of intact recombinant RANTES as a second stimulus.

25 Figure 5: Comparison of the chemotactic potency of truncated RANTES(3-68) with intact RANTES(1-68). The eosinophilic granulocyte chemotactic activity of natural (nat) and recombinant (rec) truncated RANTES, intact RANTES and synthetic MCP-3 was determined in the microchamber assay. Results represent the mean chemotactic index (CI)  $\pm$  SEM of two or more independent experiments (each performed in triplicate).

Figure 6: Desensitization of calcium mobilization by intact RANTES in CCR



with different concentrations of intact or truncated RANTES, followed by stimulation with 100 ng/ml of intact RANTES. The percentage inhibition of the  $[Ca^{2+}]_i$  increase induced by the second stimulus is shown. This percentage was calculated by comparing the response of 100 ng/ml of intact RANTES after addition of RANTES(1-68) or RANTES(3-68) with the response after stimulation with buffer (100%). Results represent the mean percentage inhibition  $\pm$  SEM of two or more experiments.

**Figure 7:** Potent inhibitory effect of RANTES (3-68) on infection of mononuclear cells by HIV-1. PHA-activated PBMC were infected with M-tropic HIV-1 Ba-L strain in the presence of various concentrations of RANTES (1-68) or RANTES (3-68) (0 to 1,000 ng/ml added at the time of infection). After ten days virus yields were monitored in the cell supernatant by a p-24 Ag ELISA (one representative experiment out of four is shown).

**Figure 8:** Effects of RANTES(1-68) and RANTES(3-68) on infection by the HIV-1 SF162 strain in PHA-activated PBMC. Virus yields were monitored 10 days after infection by a p24 Ag ELISA on the cell supernatant. Results of a representative experiment out of three are shown. \* Under the detection limit of the p24 Ag ELISA (<5 pg/ml).

**Figure 9:** Expression of CD26 on HOS.CD4.CCR5 cells, U87.CD4.CCR5 cells and freshly-isolated PBMC. The percentage (%) of CD26 positive cells is indicated in each histogram.

**Figure 10:** Effects of RANTES(1-68), RANTES(1-68) plus sCD26 (50 U/L), and RANTES(3-68) on infection of HOS.CD4.CCR5 cells by the HIV-1 BaL strain. Virus yields were monitored in the cell supernatant 8 days after infection by a p24Ag ELISA. Results of a representative experiment out of three is shown.

## EXAMPLES

### EXAMPLE 1: Amino-terminally truncated RANTES

#### *Material and Methods*

#### *Reagents*

Example 1: 1.1.1. Human Melanoblastoma cells

Antwerp and Leuven) and purified as previously described (Proost P. et al., 1996 and Proost P. et al., 1993). MCP-2, MCP-3 and GCP-2 were synthesized by Fmoc chemistry (Proost P. et al., 1995 and Wuyts A. et al., 1997), recombinant human RANTES was obtained from Peprotech (Rocky Hill, NJ) and recombinant MCP-1 was a gift from Dr. J.J. Oppenheim (NCI-NIH, Frederick, MD).

Human osteosarcoma (HOS) cells transfected with CD4 and one of the CC chemokine receptors CCR1, CCR3 or CCR5 (Deng H., et al., 1996) were grown in DMEM with glutamax. Puromycin (1 µg/ml) was added to the medium as a selection agent. All growth media (Gibco BRL/Life Technologies, Paisley, UK) were enriched with 10% FCS.

Human CD26/DPP IV was obtained from prostatesomes, prostate derived organelles, which occur freely in seminal plasma. The enzyme was purified to homogeneity as described before using ion exchange followed by affinity chromatography onto adenosine deaminase (De Meester I. et al., 1996).

#### *Incubation of chemokines with CD26/DPP IV and detection of proteolytic processing*

A 100 to 1000 molar excess of chemokine was incubated overnight with CD26/DPP IV in 100 mM Tris/HCl pH 7.7. Chemokines were separated from CD26/DPP IV by SDS-PAGE on a Tris/Tricine gel system as previously described (Proost P. et al., 1996).

Proteins were electroblotted on PVDF (polyvinylidene fluoride) membranes (Problott, Perkin Elmer, Foster City, CA) and stained with coomassie brilliant blue R250. After destaining, membranes were rinsed at least 5 times with ultrapure water (Milli Q; Millipore, Bedford, MA).

To obtain sufficient amounts of pure truncated chemokine for biological assays, about 50 µg of recombinant chemokine was treated with CD26/DPP IV and the cleavage product was acidified with 0.1 % trifluoroacetic acid (TFA). Tween 20 (0.01 %) was added to prevent the chemokines from sticking to the tubes.

Chemokines were separated from CD26/DPP IV in an acetonitrile gradient on a C-8 Aquapore RP-300 column (1 x 50 mm) (Perkin Elmer). Fractions containing proteins were analyzed by SDS-PAGE and silver stained as described.

CD26/DPP IV treated chemokines, purified by RP-HPLC or excised from PVDF blots, were NH<sub>2</sub>-terminally sequenced by Edman degradation on a pulsed liquid phase 477A/120A protein sequencer (Perkin Elmer) using *N*-methylpiperidine as a coupling base.

#### *Detection of chemotactic activity*

Chemokines were tested for their chemotactic potency on freshly isolated peripheral blood neutrophilic granulocytes (10<sup>6</sup> cells/ml) or cultured monocytic THP-1 cells (0.5 x 10<sup>6</sup> cells/ml) in the Boyden microchamber (Proost P. et al., 1996 and Proost P. et al., 1993).

After 45 min (granulocytes) or 2 h (THP-1 cells) incubation at 37 °C, the cells were fixed and stained. The cells that migrated through the 5 µm pore size polycarbonate membranes were counted microscopically in ten oil immersion fields.

The chemotactic index (C.I.) of a sample (triplicates in each chamber) was calculated as the number of cells that migrated to the test sample divided by the number of cells that migrated to control medium. In desensitization experiments, cells were incubated with biologically inactive chemokine-variants for 10 min at 37 °C before transfer to the chamber.

The percentage inhibition of the C.I. obtained by desensitization with HBSS-treated control cells was calculated for the evaluation of chemotaxis desensitization.

#### *Detection of intracellular Ca<sup>2+</sup> concentrations*

Intracellular Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>i</sub>) were measured as previously described (Wuyts A., et al., 1997). Briefly, purified cells were incubated with the fluorescent indicator fura-2 (2.5 µM fura-2/AM, Molecular Probes Europe BV, Leiden, The Netherlands) and 0.01 % Pluronic F-127 (Sigma, St. Louis, MO).

Cells were washed twice, resuspended in HBSS with 1 mM Ca<sup>2+</sup>

luminescence spectrophotometer (Perkin Elmer). Upon excitation at 340 and 380 nm, fluorescence was detected at 510 nm. The  $[Ca^{2+}]_i$  was calculated from the Grynkiewicz equation (Grynkiewicz G. et al., 1985).

In order to determine  $R_{max}$ , the cells were lysed with 50  $\mu$ M digitonin. Subsequently, the pH was adjusted to 8.5 with 20 mM Tris and  $R_{min}$  was obtained by addition of 10 mM EGTA to the lysed cells. The  $K_d$  used for calibration was 224 nM. For desensitization experiments, cells were first stimulated with buffer or chemokine at different concentrations. As a second stimulus, chemokines were added at a concentration inducing a significant increase in the  $[Ca^{2+}]_i$  after prestimulation with buffer. The percentage inhibition of the  $[Ca^{2+}]_i$ -increase in response to the second stimulus by prestimulation of the cells was calculated.

#### *Inhibition of HIV-1 infection*

The HIV-1 M-tropic strains BaL and SF162 were obtained through the MRC AIDS reagent project (Herts, UK). Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by density gradient centrifugation (5,23) and stimulated with PHA at 1  $\mu$ g/ml (Sigma, Bornem, Belgium) for 3 days at 37°C. The activated cells (PHA-stimulated blasts) were washed three times with PBS, and infected with a virus as described previously (Schols D. et al., 1997). HIV-1 infected or mock-infected PHA-stimulated blasts were cultured in the presence of 25 U/ml of IL-2 and varying concentrations of RANTES (1-68) or RANTES (3-68). Cell supernatant was collected at day 10 and HIV-1 core antigen in the supernatant was analysed by a p-24 Ag ELISA kit (DuPont/NEN Life Science Products, Brussels, Belgium).

#### *Results*

##### *Identification and biological characterization of natural, NH<sub>2</sub>-terminally truncated RANTES.*

A different NH<sub>2</sub>-terminally truncated form of human GCP-2 has been previously isolated (Proost P. et al., 1993). The least truncated GCP-2-form was cleaved beyond

... standard purification

procedure, the C-C chemokine RANTES was purified from peripheral blood leukocytes or sarcoma cells (Proost P. et al., 1996).

In particular, conditioned media from MG-63 or Malavu sarcoma cells induced with a cytokine mixture were fractionated to isolate natural chemokine variants. The chemokines were purified by subsequent antibody or heparin affinity chromatography, cation-exchange chromatography (mono S FPLC) and RP-HPLC, and immunoreactive forms were detected by specific chemokine ELISAs. On the cation-exchange column, IL-8 was found to elute in close proximity of RANTES (between 0.7 and 0.75 M NaCl). Nevertheless, both chemokines were separated from each other by RP-HPLC (RANTES and IL-8 eluting at 27.5% and 30% acetonitrile, respectively). Amino acid sequence analysis of the pure proteins confirmed that IL-8 occurred in different NH<sub>2</sub>-terminally truncated forms, which were previously isolated on the basis of their chemotactic activity (Van Damme J. et al., 1989). However, for RANTES only one single form was isolated, which was missing two NH<sub>2</sub>-terminal residues compared to intact RANTES. In view of its predominant appearance, this RANTES(3-68) was analyzed in more detail to verify its chemotactic activity for monocytes and eosinophils. In particular, RANTES(3-68) was tested for chemotactic and/or intracellular Ca<sup>2+</sup>-releasing activity and their biological potency was compared with that of the respective intact chemokines.

NH<sub>2</sub>-terminal deletion of two residues from RANTES resulted in considerably decreased monocyte chemotactic and Ca<sup>2+</sup>-releasing activities. Compared to intact natural RANTES (minimal effective dose of 3-10 ng/ml), natural RANTES(3-68) was totally inactive when tested at concentrations as high as 300 ng/ml in the Boyden microchamber (Figure 2). In addition, 10 times higher concentrations of natural RANTES(3-68), compared to RANTES(1-68), were necessary to obtain a similar Ca<sup>2+</sup>-response (Figure 3).

#### *CD26/DPP IV removes the NH<sub>2</sub>-terminal dipeptides of chemokines*

In order to investigate whether the aminopeptidase CD26/DPP IV could be responsible for the NH<sub>2</sub>-terminal truncation of RANTES, the intact chemokine was

incubated with CD26/DPP IV. Blotted to PVDF membranes, stained with

treatment of RANTES resulted in the removal of the NH<sub>2</sub>-terminal dipeptides. Parallel incubation of chemokine with buffer without CD26/DPP IV had no effect.

Since other chemokines contained the consensus sequence for CD26/DPP IV cleavage and since the NH<sub>2</sub>-terminus of MCPs was shown to be crucial for biological activity (Gong J. et al., 1996 and Gong J. et al., 1995), MCP-1, MCP-2 and MCP-3 were also incubated with CD26/DPP IV.

After treatment, MCPs were blotted on PVDF membranes and Coomassie blue stained to confirm that a sufficient amount of protein was recovered for Edman degradation.

However, no NH<sub>2</sub>-terminal sequence could be detected, indicating that CD26/DPP IV does not alter the NH<sub>2</sub>-terminus of MCPs which is blocked for Edman degradation by a pyroglutamic acid.

#### *Comparison of the biological activity of intact and CD26/DPP IV-treated RANTES.*

Similar to natural RANTES(3-68), C-8 RP-HPLC purified, CD26/DPP IV-treated recombinant RANTES was inactive in Boyden microchamber chemotaxis experiments when used at concentrations up to 1 µg/ml, while a significant monocyte chemotactic response was detected with intact recombinant RANTES from 30 to 100 ng/ml onwards (Figure 2).

When the truncation effect was tested in the Ca<sup>2+</sup>-mobilization assay, RANTES(3-68) induced a low but significant increase at 100 ng/ml. Intact RANTES, however, was already active at 10 ng/ml (Figure 3). In conclusion, although only two NH<sub>2</sub>-terminal residues were removed, the monocyte chemotactic and Ca<sup>2+</sup>-mobilizing potency of RANTES decreased 10 to 100-fold.

25

#### *RANTES(3-68) is a natural chemotaxis antagonist for intact RANTES*

In view of the inactivity of RANTES(3-68) in monocyte chemotaxis experiments, we tested whether this truncated RANTES might act as an antagonist. RANTES(3-68), at 1 µg/ml, almost completely (82 %) desensitized for the chemotactic effect of 100

When a 3-fold excess of RANTES(3-68) was added to the upper well, chemotaxis of THP-1 cells towards intact RANTES was inhibited by about 50-70 %. RANTES(3-68) at 300 ng/ml could still inhibit about 30 % of the chemotactic response towards an equal concentration of intact RANTES.

5        In  $\text{Ca}^{2+}$ -mobilization experiments with THP-1 cells (Figure 4), 30 ng/ml of intact RANTES could desensitize for the effect of 30 ng/ml of intact RANTES for  $39 \pm 5$  %. About ten-fold higher concentrations of RANTES(3-68) were necessary to obtain the same amount of desensitization. However, at 300 ng/ml, RANTES(3-68) by itself gave a significant  $\text{Ca}^{2+}$ -response. This  $\text{Ca}^{2+}$ -response was comparable to the response obtained  
10        with 30 ng/ml of intact RANTES.

-15-  
TABLE I

*RANTES(3-68) desensitizes monocyte chemotaxis induced by RANTES(1-68)<sup>1</sup>*

Chemokine(ng/ml)		Chemotactic response (CI)					
Lower well	Upper well						% inhibition
RANTES (1-68)	RANTES (3-68)	A	B	C	D	mean $\pm$ SEM	mean $\pm$ SEM
300	1000	12.5	7.5	27.5	50.5	25 $\pm$ 10	67 $\pm$ 8
	300	22.0	20.5	72.5	79.5	49 $\pm$ 16	31 $\pm$ 13
	0	41.0	46.0	71.5	97.0	64 $\pm$ 3	0
100	1000	4.0	3.0	13.5	11.0	8 $\pm$ 3	82 $\pm$ 4
	300	7.5	7.0	29.0	33.0	19 $\pm$ 7	53 $\pm$ 11
	0	24.0	21.5	50.0	44.5	35 $\pm$ 7	0

5

<sup>1</sup> Results represent the chemotactic index (C.I.) of four (A to D) independent experiments (including mean  $\pm$  SEM) and the percentage (%) inhibition (mean  $\pm$  SEM of the % inhibition of the four experiments) of the chemotactic response towards RANTES(1-68) after preincubation of the THP-1 cells with inactive RANTES(3-68) or buffer.

10

*Impaired chemotactic activity of RANTES(3-68) for human monocytes and eosinophils*

In Table II the chemotactic potency of natural RANTES(3-68) is compared with that of the monocyte chemotactic protein MCP-3 and intact RANTES(1-68). It can be seen that MCP-3 and RANTES(1-68) are still chemotactic for freshly isolated peripheral blood monocytes at 3 ng/ml and 30 ng/ml, respectively, whereas natural RANTES(3-68) remained inactive at 100 ng/ml.

15

The reduced chemotactic potency of this natural variant, was confirmed with recombinant RANTES(3-68). Although weakly chemotactic for monocytes (at 1  $\mu$ g/ml), purified recombinant RANTES(3-68) showed a specific activity which is 10-fold lower than that of intact recombinant RANTES.

20

Finally, the chemotactic potency of RANTES(3-68) was verified on human



MCP-3 (Figure 5). Similar to monocytes, eosinophil migration was only stimulated by RANTES(3-68) at 1 µg/ml.

TABLE II

5 *Comparison of the monocyte chemotactic activity of RANTES(3-68) with RANTES(1-68) and MCP-3*

monocyte chemotactic activity <sup>a)</sup>				
conc. (ng/ml)	MCP-3	natRANTES (3-68)	recRANTES (1-68)	recRANTES (3-68)
1000	— <sup>b)</sup>	—	3.6 ± 0.8 (6)	3.3 (1)
300	6.0 ± 1.2 (6)	—	—	—
100	—	1.1 ± 0.1 (3)	3.3 ± 0.4 (6)	1.0 (1)
30	6.9 ± 1.0 (6)	1.7 ± 0.2 (3)	—	—
10	—	1.9 ± 0.6 (3)	1.9 ± 0.4 (6)	< 1.0 (1)
3	4.1 ± 0.4 (6)	—	—	—

<sup>a)</sup> mean chemotactic index (CI) ± SEM (n) on freshly isolated peripheral blood monocytes.

<sup>b)</sup> not determined

*RANTES(3-68) signals and desensitizes for RANTES(1-68) through CCR5, but not through CCR1 and CCR3*

15 To explain the reduced chemotactic activity of RANTES(3-68), the capacity of this chemokine variant to bind and signal through the known receptors used by RANTES was verified.

HOS cells transfected with the chemokine receptors CCR1, CCR3 or CCR5 were used in a signaling assay measuring increases in the intracellular calcium concentration. At concentrations up to 300 ng/ml, RANTES(3-68) did not increase the [Ca<sup>2+</sup>]<sub>i</sub> in HOS cells transfected with CCR1 (Table III) or CCR3 (data not shown), whereas 30 ng/ml and 100 ng/ml of intact RANTES was sufficient to induce an increase in [Ca<sup>2+</sup>]<sub>i</sub> in CCR1 and CCR3 transfectants, respectively.

However, both intact and truncated RANTES were able to induce a significant

CCR5-transfected cells with a 3 to 10-fold excess of either RANTES(3-68) or intact RANTES, an equal inhibition (about 75%) of the  $[Ca^{2+}]_i$  rise by a subsequent challenge with intact RANTES (100 ng/ml) was obtained (Figure 6).

In contrast, 300 ng/ml of RANTES(3-68) only marginally desensitized the calcium response of CCR1 and CCR3-transfected cells to 100 ng/ml of intact RANTES, whereas a 3-fold excess of intact RANTES as first stimulus almost completely inhibited the  $[Ca^{2+}]_i$  rise in these cells by subsequent RANTES(1-68). It must be concluded that removal of two  $NH_2$ -terminal residues from RANTES has a significant impact on signal transduction in that the chemokine receptors CCR1 and CCR3 are no longer functionally recognized. Therefore, the impaired chemotactic potency of RANTES(3-68) can be explained by its inability to function through CCR1 and CCR3. In contrast, RANTES(3-68) fully retained the CCR5 signalling characteristic of intact RANTES. RANTES(3-68) can be anti-inflammatory by competing with intact RANTES, but may still function as an HIV-inhibitor by retaining its capacity to bind CCR5.

TABLE III

*Calcium mobilization by RANTES forms in CCR1 and CCR5 transfectants*

chemokine	conc. (ng/ml)	increase in $[Ca^{2+}]_i$ (nM) <sup>a)</sup>	
		CCR1	CCR5
RANTES(1-68)	300	133 ± 5 (3)	96 ± 1 (2)
	100	100 ± 28 (3)	60 ± 4 (2)
	30	25 ± 8 (3)	24 ± 2 (2)
	10	<15 (2)	<15 (1)
RANTES(3-68)	300	19 ± 9 (3)	119 ± 5 (2)
	100	<15 ± 0 (3)	76 ± 4 (2)
	30	<15 (2)	56 ± 13 (2)
	10		<15 (1)

<sup>a)</sup> the mean increase in  $[Ca^{2+}]_i$  in nM ± SEM of two or more independent experiments is shown.

*Inhibition of CC chemokine-induced chemotaxis by RANTES(3-68) in human monocytic cells*

To verify whether inhibition of CC chemokine signaling by RANTES(3-68) also occurred in monocytic cells, inhibition experiments were conducted in THP-1 cells. It was evidenced that RANTES(3-68) showed a 10-fold reduction in potency to increase the  $[Ca^{2+}]_i$  in monocytic cells compared to intact RANTES (data not shown). In addition, the chemotactic effect of intact RANTES (30 ng/ml) on monocytic cells was inhibited (71%) by incubating the test cells with 300 ng/ml RANTES(3-68) as shown in Table IV.

Furthermore, RANTES(3-68) reduced the chemotactic response to other CC chemokines, including monocyte chemotactic protein-3 (MCP-3) (67%), macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) (61%) and MIP-1 $\beta$  (80%).

This illustrates that RANTES(3-68) functions as a broad spectrum inhibitor of monocytic cell migration induced by other CC chemokines.

TABLE IV

*Inhibition of monocytic cell chemotaxis towards CC chemokines by RANTES(3-68)*

inhibition of THP-1 cell chemotaxis				
chemokine <sup>a)</sup>	conc. (ng/ml)	buffer <sup>b,c)</sup>	RANTES(3-68) <sup>b,c)</sup>	% inhibition <sup>d)</sup>
RANTES	30	19.0 $\pm$ 6.6	3.7 $\pm$ 0.6	71 $\pm$ 16
MCP-3	30	48.5 $\pm$ 9.3	24.9 $\pm$ 2.0	45 $\pm$ 10
MCP-3	3	7.6 $\pm$ 2.5	3.1 $\pm$ 0.8	67 $\pm$ 13
MIP-1 $\alpha$	30	6.2 $\pm$ 2.4	3.0 $\pm$ 1.1	61 $\pm$ 22
MIP-1 $\beta$	300	4.3 $\pm$ 1.0	1.9 $\pm$ 0.6	80 $\pm$ 12
control		1.5 $\pm$ 0.5	1.0 $\pm$ 0.5	

a) RANTES, MCP-3, MIP-1 $\alpha$ , MIP-1 $\beta$  and buffer were added as chemoattractants to the lower wells of the microchamber.

b) the upper wells of the microchamber were filled with THP-1 cells preincubated (10 min, 37°C) with 300 ng/ml RANTES(3-68) or with buffer.

c) mean CI  $\pm$  SEM of four independent experiments.

*CD26-specific truncation of RANTES is necessary for its antiviral activity.*

The effects of the different forms of RANTES were first evaluated against two different M-tropic HIV-1 strains (BaL and SF162) in human PBMC derived from healthy blood donors. The  $IC_{50}$  of the intact RANTES(1-68) against the BaL strain was 3.4 nM and for RANTES(3-68) the  $IC_{50}$  was 0.39 nM. The  $IC_{90}$  value for RANTES(1-68) was 71 nM, which was about 10-fold the  $IC_{90}$  for RANTES(3-68). Against the SF162 strain when evaluated in PBMC, RANTES(1-68) ( $IC_{50}$ : 23 nM;  $IC_{90}$  : 95 nM) was more than 10-fold less active than RANTES(3-68) ( $IC_{50}$ : 2 nM;  $IC_{90}$  : 8.2 nM) (Table V). The concentration-dependent effects of both chemokines at concentrations ranging from 133 down to 0.2 nM against HIV-1 SF162 replication in PBMC are shown in Figure 8. A concentration of 5.2 nM RANTES(3-68) was clearly effective in reducing virus replication, whereas RANTES(1-68) was inactive at this concentration. No difference in antiviral activity was noticed between intact RANTES obtained from PeproTech or R&D Systems.

The striking difference in antiviral activity between the two forms of RANTES became even more apparent when tested in the human CCR5 transfected cells. In U78.CD4.CCR5 cells, the  $IC_{50}$  for RANTES(1-68) and RANTES(3-68) against the BaL strain was 21 nM and 0.65 nM, respectively. The  $IC_{90}$  for RANTES(1-68) was more than 133 nM, whereas the  $IC_{90}$  for RANTES(3-68) was 63 nM. Also in Table V, it is shown that RANTES(1-68) was virtually inactive in the HOS.CD4.CCR5 cells ( $IC_{50}$  > 133 nM), whereas RANTES(3-68) is a potent inhibitor of HIV-1 BaL replication in these cells ( $IC_{50}$ : 5.5 nM). However, no  $IC_{90}$  values were reached for both forms of RANTES in these cells.

*The antiviral activity of RANTES is dependent on the presence of membrane bound or soluble CD26 (sCD26).*

The CD26 expression on the two different CCR5-transfected cell lines and on freshly isolated PBMC was evaluated. HOS transfectants were negative for CD26

subpopulation of freshly isolated PMBC was found to be strongly positive for CD26 expression (Figure 9).

The concentration-dependent effect of RANTES(1-68) and RANTES(3-68) on viral p24 Ag production by the BaL strain in HOS.CD4.CCR5 transfected cells in presence of sCD26 is shown in Figure 10. Addition of sCD26, together with RANTES(1-68) at the start of the HIV infection, significantly enhanced the antiviral activity of the intact RANTES in HOS.CD4.CCR5 cells. When sCD26 at 50 U/l was added together with RANTES, an  $IC_{50}$  of 13 nM of RANTES was obtained. The addition of sCD26 alone had no effect on virus replication. The addition of sCD26 to RANTES(3-68) did also not change the antiviral activity of RANTES(3-68) (data not shown). Thus, the presence of CD26 is essential for intact RANTES to become antivirally active.

*Amino terminal truncation of natural MIP-1 $\alpha$  does not affect its anti-HIV-1 chemotactic and  $Ca^{2+}$  mobilising activity.*

Since the majority of natural MIP-1 $\alpha$  is  $NH_2$  terminally truncated (four amino acids), we investigated whether this truncated MIP-1 $\alpha$ (5-70) had an altered HIV-1 inhibitory capacity. In contrast with the results obtained for RANTES, no significant differences were detected for the  $IC_{50}$  values of intact MIP-1 $\alpha$  and MIP-1 $\alpha$ (5-70) in PMBC or CCR5-transfected cells (Table V, Figure 8). In addition, intact MIP-1 $\alpha$  and truncated MIP-1 $\alpha$ (5-70) were compared in chemotaxis and intracellular  $Ca^{2+}$ -mobilization assays on THP-1 monocytic cells. Table VI demonstrates that the minimal effective dose of MIP-1 $\alpha$ (5-70) inducing a rise in the  $[Ca^{2+}]_i$  was only slightly lower than intact MIP-1 $\alpha$ . Furthermore, although maximal migration obtained with 0.13 nM in the chemotaxis assay was higher for intact MIP-1 $\alpha$ , the minimal affective concentrations of both MIP-1 $\alpha$  isoforms were rather similar. Taken together, it must be concluded that  $NH_2$  terminal processing of MIP-1 $\alpha$  in contrast to RANTES, only minimally weakens its inflammatory and anti-HIV-1 activity.

**TABLE V.** Anti-HIV-1 activity of RANTES and MIP-1 $\alpha$  in PHA-stimulated PMBC, U87.CD4.CCR5 cells.

RANTES(1-68)		RANTES(3-68)		MIP-1 $\alpha$ (1-70)		MIP-1 $\alpha$ (5-70)	
IC <sub>50</sub>	IC <sub>90</sub>	IC <sub>50</sub>	IC <sub>90</sub>	IC <sub>50</sub>	IC <sub>90</sub>	IC <sub>50</sub>	IC <sub>90</sub>
<b>PMBC</b>							
<b>BaL</b>							
3.4	71	039	6.9	1.9	62	1.6	13
<b>SF162</b>							
23	95	2.0	8.2	3.1	30	3.6	32
<b><u>U87.CD4.CCR5</u></b>							
<b>BaL</b>							
21	>130	0.65	63	ND	ND	ND	ND
<b><u>HOS.CD4.CCR5</u></b>							
<b>BaL</b>							
>130	>130	5.5	>130	32	>130	21	>130

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Virus yield was monitored in the cell-free supernatant 8-12 days after infection by viral p24 Ag ELISA. The mean IC<sub>50</sub>s and IC<sub>90</sub>s (in nM) are shown. The data represent the means of two to four independent experiments. The value marked by ">130" indicates that 50% or 90% inhibition is not achieved at 130 nM. ND, not done.

**TABLE VI** Lack of difference in biological potency between intact and truncated MIP-1 $\alpha$ .

Chemokine	Chemotaxis*		Increase in $[Ca^{2+}]_i^{\dagger}$	
	nM	C.I	nM	nM $[Ca^{2+}]_i$
MIP-1 $\alpha$ (1-70)	1.3	$8.5 \pm 3.3$	3.9	240/195
	0.13	$22.2 \pm 4.4$	0.39	120/130
	0.013	$5.7 \pm 1.6$	0.34	30/14
	0.0013	$4.0 \pm 3.1$		
MIP-1 $\alpha$ (5-70)	1.3	$14.2 \pm 0.4$	4.0	196/178
	0.13	$11.4 \pm 2.9$	0.4	71/33
	0.013	$3.8 \pm 1.4$	0.04	10/<10
	0.0013	$2.1 \pm 0.6$		

\* Migration of monocyte THP-1 cells through 5.0  $\mu$ m pores in the microchamber. Results are mean  $\pm$  of three independent experiments.

$\dagger$  Detection of the  $[Ca^{2+}]_i$  increase in THP-1 cells. Results of two independent experiments are shown.

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